The 3’ end of the foot-and-mouth disease virus genome establishes two distinct long-range RNA–RNA interactions with the 5’ end region

Paula Serrano,1 Miguel Rodriguez Pulido,2 Margarita Sáiz1,2 and Encarnacion Martínez-Salas1

1Centro de Biología Molecular Severo Ochoa, CSIC-UAM, 28049 Madrid, Spain
2CISA-INIA, Valdeolmos, 28130 Madrid, Spain

The untranslated regions (UTRs) of the foot-and-mouth disease virus (FMDV) genome contain multiple functional elements. In the 5’ UTR, the internal ribosome entry site (IRES) element governs cap-independent translation initiation, whereas the S region is presumably involved in RNA replication. The 3’ UTR, composed of two stem–loops and a poly(A) tract, is required for viral infectivity and stimulates IRES activity. Here, it was found that the 3’ end established two distinct strand-specific, long-range RNA–RNA interactions, one with the S region and another with the IRES element. These interactions were not observed with the 3’ UTR of a different picornavirus. Several results indicated that different 3’ UTR motifs participated in IRES or S region interactions. Firstly, a high-order structure adopted by both the entire IRES and the 3’ UTR was essential for RNA interaction. In contrast, the S region interacted with each of the stem–loops. Secondly, S–3’ UTR interaction but not IRES–3’ UTR interaction was dependent on a poly(A)-dependent conformation. However, no other complexes were observed in mixtures containing the three transcripts, suggesting that these regions did not interact simultaneously with the 3’ UTR probe. Cellular proteins have been found to bind the S region and one of these also binds to the 3’ UTR in a competitive manner. Our data suggest that 5’–3’ end bridging through both direct RNA–RNA contacts and RNA–protein interactions may play an essential role in the FMDV replication cycle.

INTRODUCTION

The foot-and-mouth disease virus (FMDV) genome consists of a positive-sense RNA of about 8500 nt encoding a polyprotein, which is processed into the mature viral products. In common with all picornaviruses, the 5’ end of the viral RNA is linked covalently to the viral protein VPg and the 3’ end is polyadenylated. 5’ and 3’ untranslated regions (UTRs) of approximately 1300 and 100 nt, respectively, flank the single open reading frame (Belsham & Martinez-Salas, 2004). The FMDV 5’ UTR displays the most complex organization among picornavirus, comprising the S region, a poly(C) tract, several pseudoknots, the cis-acting replication element (cre) and the internal ribosome entry site (IRES). The S region, spanning about 360 nt at the 5’ terminus of the viral RNA, is predicted to form a long hairpin structure (Escarmis et al., 1992; Witwer et al., 2001). An important role in replication is assumed for this region, although direct evidence for protein interactions or the molecular mechanisms involved is still lacking. In poliovirus (PV), the 5’ end of the genome adopts a cloverleaf structure that has been shown to interact with the cellular and viral proteins poly(rC)-binding protein 2 (PCBP2) and 3CD proteinase, respectively (Gamarnik & Andino, 1997; Parsley et al., 1997). A role for these interactions has been proposed in regulating the switch from translation to replication (Gamarnik & Andino, 1998). A second role in RNA circularization has been also suggested, as PCBP2 binds to the poly(A)-binding protein (PABP), which is known to interact with the viral poly(A) tail (Herold & Andino, 2001).

The FMDV IRES (about 450 nt), mediating cap-independent translation of the viral RNA, has been studied extensively (Martinez-Salas & Fernández-Miragall, 2004). The essential domains, RNA secondary structure and interaction with cellular proteins have been described (Fernández-Miragall & Martinez-Salas, 2003; Fernández-Miragall et al., 2006; López de Quinto & Martinez-Salas, 1997, 2000; Pilipenko et al., 2000; Stassinopoulos & Belsham, 2001; Walter et al., 1999). Secondary structure analysis of the FMDV 3’ UTR sequence predicts the presence of two stable stem–loops. This region is strictly required for replication and infectivity (Sáiz et al., 2001). In other picornaviruses, the 3’ UTR is organized into two stem–loops that adopt a quasi-globular organization (Melchers et al., 2000) and constitute essential determinants of virus replication (Brown et al., 2005; Dobrikova et al., 2003; Melchers et al., 1997).
Several strategies have been proposed for 5′–3′-end contacts in positive-strand RNA viruses, including rotaviruses, picornaviruses and pestiviruses, all involving RNA-binding proteins (Herold & Andino, 2001; Isken et al., 2003, 2004; Piron et al., 1998; Vende et al., 2000). To our knowledge, no data regarding genome circularization based on direct RNA–RNA interaction have been reported for picornaviruses. A direct RNA–RNA cyclization mediated by genomic complementary sequences (5′ and 3′ CS) present in the capsid-coding region and the 3′ UTR, respectively, has been proposed for flaviviruses (Corver et al., 2003; Khromykh et al., 2001, 2003; Shurtleff et al., 2001). A recent report using atomic force microscopy showed that dengue virus RNA circularizes through RNA–RNA interactions in the absence of proteins (Alvarez et al., 2005). An additional pair of complementary sequences located at the 5′ end upstream of the initiator AUG and at the 3′ end was also identified as necessary, together with the 5′ and 3′ CS, for cyclization. The requirement for Mg<sup>2+</sup> ions for 5′–3′ associations strongly suggests tertiary interactions in the RNA.

We have shown previously that the FMDV 3′ UTR stimulates IRES activity, even in the absence of a poly(A) tail (Lopez de Quinto et al., 2002), suggesting a functional link between the IRES, which resides at the 5′ end, and the 3′ end of the genomic viral RNA. This interaction could take place through direct RNA–RNA contacts, through protein bridges mediating RNA circularization or both. In this work, we conducted studies aimed at understanding the involvement of the 3′-terminal region in viral IRES stimulation and replication efficiency. We have shown here that regions located at the 5′ and 3′ ends of the FMDV genome interact through strand-specific RNA–RNA contacts. Two different elements, S and IRES, present at the 5′ end, interacted with the 3′ UTR in a dose-dependent manner. No interaction was observed with the 3′ UTR of swine vesicular disease virus (SVDV), a picornavirus that produces similar symptoms in infected animals. The regions involved in the RNA–RNA contacts were dissected and the resulting interaction patterns analysed. A high-order RNA structure adopted by both the complete IRES and the 3′ UTR was essential for RNA interaction, whereas the S region could interact with each of the 3′ UTR stem–loops. Additionally, we detected specific cellular proteins interacting with the S region. One of these, p47, competed for binding to the 3′ UTR. Our data on FMDV 5′–3′-end bridging, involving both direct RNA–RNA contacts and RNA–protein interactions, provide a mechanistic basis for translation stimulation, and possibly replication, of the viral RNA.

**METHODS**

**Plasmids.** cDNAs corresponding to the S regions (nt 1–367) of FMDV strains O1K and CS-8 viral RNAs (Escarmis et al., 1992) were amplified by RT-PCR using primers Sac-S-R-C (5′-GGAACGAGCTCTGAAGGGCGGGTTTCGGGTGAC-3′), Sac-S-R-O (5′-GGAAAGCAGCTCTGAAAGGGGCGGGCTGGGTGAC-3′) and Sac-S-L-O-C (5′-GGAAACGAGCTCTGAAAGGGGCGGGCTGGGTGAC-3′), which introduced flanking SacI restriction sites (underlined). PCR products were digested with SacI and cloned in both orientations into Bluescript II SK+, which had been linearized previously with SacI and dephosphorylated.

Plasmid subTAG containing the complete FMDV 3′ UTR (FMDV-TAG) has been described previously (Lopez de Quinto et al., 2002). pSL1 and pSL2 plasmids carried 3′ end sequences corresponding to the SL1 and SL2 predicted stem–loops. These constructs were made in two consecutive steps. Firstly, the full-length clone pDM FMDV-O1K (Sáiz et al., 2001) was modified by recombinant PCR to delete the SL1 and SL2 regions precisely (nt 4–33 and 50–82 from the viral RNA stop codons, respectively). Secondly, MluI–HpaI fragments resulting from these clones, containing the modified 3′ end regions, were filled in with T4 DNA polymerase and ligated into Bluescript II SK+, which had previously been linearized with SacI, blunted and dephosphorylated. Plasmid subSVDV contained the 3′ UTR of SVDV plus a 58 nt poly(A) tail and was derived from construct pCHIM (Sáiz et al., 2001) by excision of the AvrII–HpaI fragment, blunted-end and cloning into Bluescript II SK+ that had been treated as above. In all cases, the nucleotide sequence of each cDNA was determined using automatic sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit; Perkin Elmer).

**pGEM-based clones.** cDNAs containing the full-length FMDV IRES sequence or the different domains have been described previously (López de Quinto et al., 2001; Ramos & Martínez-Salas, 1999).

**In vitro transcription.** Prior to RNA synthesis, plasmids were linearized using NotI for the generation of S and 3′ UTR transcripts and using XhoI for the IRES transcript. DNA templates lacking the 58 nt poly(A) tail [A(A) derivatives] were made by linearization of the corresponding clones with EcoRV. Transcription was performed for 1 h at 37°C using 50 U T7 or T3 RNA polymerase in the presence of 0.5–1 µg linearized DNA template, 50 mM DTT, 0.5 mM NTPs and 20 µRNAs. When needed, RNA transcripts were labelled uniformly using [α-<sup>32</sup>P]CTP (400 Ci (14.8 TBq) mmol<sup>-1</sup>). Reactions were incubated for 15 min with 1 U RQI DNase and unincorporated isotope was eliminated in MicroSpin G-50 columns.

**Gel-shift assays.** For RNA–RNA interactions, uniformly <sup>32</sup>P-labelled transcripts were incubated independently at 95°C for 3 min, transferred to ice and mixed with the indicated unlabelled RNAs denatured as above, in 50 mM sodium cacodylate (pH 7.5), 300 mM KCl and 1 mM MgCl<sub>2</sub> (Ferrandon et al., 1997). Poly(A) probe was 3′-end-labelled using 1 µM cytidine 3′,5′-bis[<sup>32</sup>P]phosphate and RNA ligase (New England Biolabs), as recommended by the manufacturers. For controls, the antisense version of the indicated RNA was synthesized and used as a strand specificity control; tRNA was used as an unrelated negative control. RNA–RNA complexes were allowed to form for 30 min at 37°C and immediately analysed by electrophoresis in native polyacrylamide gels supplemented with 2.5 µM MgCl<sub>2</sub> (Fedor & Uhlenbeck, 1990; Fernández-Miragall et al., 2006; Paillart et al., 1996). Electrophoresis was performed at 3°C for 33 min at 180 V in TBM buffer [45 mM Tris/HCl (pH 8.3), 43 mM boric acid, 0.1 mM MgCl<sub>2</sub>]. In all cases, dried gels were analysed by autoradiography and exposed to a phosphorimager plate to quantify the intensity of the retarded bands, as described previously (Ramos & Martínez-Salas, 1999).

**RNA–protein interaction assays.** S10 extracts from BHK-21 or HeLa cells were prepared as described previously (López de Quinto & Martínez-Salas, 2000). UV cross-linking assays were performed using 20–40 µg native proteins present in S10 extracts and 0.03 pmol of the specific 32P-labelled RNA. In competition assays, the binding buffer was adjusted to 5 mM HEPES (pH 7.4), 20 mM KCl, 2.5% glycerol, 0.5% NP-40, 0.5 mM DTT, 2.5 mM MgCl<sub>2</sub>, as described previously (Isken et al., 2003). In all cases, the RNA–protein mixture was digested with an excess of RNase A for 30 min at 37°C, followed by
the addition of SDS-loading buffer, heating for 2 min at 95 °C and electrophoresis through SDS-polyacrylamide gels. The dried gels were used to visualize the 32p-labelled proteins by autoradiography. Immunoprecipitation and Western blot assays were carried out as described previously (López de Quinto et al., 2001).

RESULTS

Strand-specific distant RNA–RNA interactions in the FMDV genome

The FMDV genome contains a long, highly structured 5′ UTR, which, together with the 3′ UTR, contains regions essential for several steps during the virus replication cycle. In a previous study, we showed specific stimulation of FMDV IRES activity by sequences in the 3′ UTR (Lopez de Quinto et al., 2002). 3′ UTR sequences have also been shown to be essential for FMDV infectivity (Sáiz et al., 2001).

Here, we investigated the potential involvement of 3′ and 5′ UTR sequences in establishing a functional link between the ends of the FMDV genome. To this end, transcripts encompassing the S region, the IRES element and different versions of the 3′ UTR were generated (Fig. 1a). In order to test the occurrence of direct interactions between the individual RNA elements, a uniformly labelled transcript corresponding to the 3′ UTR sequence was incubated with either the IRES or the S region (Fig. 1b). A specific retarded complex was detected in native gels following incubation with the IRES element. An intense complex of higher mobility was also detected following incubation with the S region. Lack of interaction with the S antisense transcript confirmed the strand specificity of these complexes (Fig. 1b). A truncated S transcript of 135 nt, lacking its hairpin structure, was unable to interact with the 3′ UTR (data not shown). Furthermore, no interaction was observed with unrelated control RNAs (Fig. 1b).

Considering the RNA–RNA complex intensity observed between the 3′ UTR probe and the S region, we asked whether the inverse assay would lead to the same result. Thus, a uniformly labelled S probe was incubated with unlabelled 3′ UTR RNA (Fig. 1c). Again, an intense, retarded complex was observed, in agreement with the data in Fig. 1(b). Therefore, interaction between the 3′ end and the S region occurred irrespective of the RNA used as the probe. However, the S region did not interact with the IRES element (the faint bands observed had a mobility different from those expected for an S–IRES complex). This result indicated that the 3′ UTR was mediating two distinct, specific interactions with different regions in the 5′ UTR.

The intensity of retarded complexes was dependent on the concentration of unlabelled RNA present in the binding assay. At the highest RNA concentration, about 65 % of the probe was retarded in the 3′ UTR–S interaction (Fig. 2a). The same efficiency of 3′ UTR–S interaction was observed, irrespective of the FMDV isolate (C-S8 or O1K) used to obtain the S region (data not shown). The sequence of this region differs by about 12% of its nucleotide sequence between these isolates (Escarmis et al., 1992), although its predicted RNA structure is conserved.

The reverse assay, using labelled S probe and unlabelled 3′ UTR transcript, was similarly dependent on RNA concentration (Fig. 2b). Thus, the two ends of the genomic FMDV RNA, the S region and the 3′ end, could interact with each
stimulation of IRES activity mediated by specific viral RNA 3’ end sequences (Lopez de Quinto et al., 2002). Deletion studies aimed at mapping the RNA-binding site(s) in the IRES indicated that none of the separate IRES domains retained the ability to interact with the 3’ UTR probe, with the exception of a faint binding capacity to domains 4–5 (Fig. 3a). The 3’ UTR transcript used in this work contained a tract of 58 adenines, as present in the parental FMDV infectious clone (Sáiz et al., 2001). Deletion of the poly(A) tail in transcript 3’ΔΔ(A) did not impair its binding capacity to the entire IRES (Fig. 3b). In agreement with results using the complete 3’ UTR, only domains 4–5 appeared to retain a faint binding capacity to this probe.

According to computer prediction programs, and consistent with phylogenetic conservation, the 3’ UTR is composed of two stem–loops separated by a connecting region of 12 residues (Fig. 4a). A secondary structure comparison of different FMDV isolates belonging to O, A, C, Asia 1 and Sat 1-3 serotypes showed strong conservation of the stem–loops, with most of the nucleotide variability accumulated either in the connecting region or in the unpaired bulges of each stem–loop. Deletions or insertions were scarce.

Two different constructs, SL1 and SL2, were generated in order to map the IRES-interacting residues. Each of these constructs was designed to delete one of the conserved stem–loops precisely, leaving the connecting region and the poly(A) tract intact. Neither SL1 nor SL2 transcripts possessing an intact poly(A) tail interacted with the IRES (data not shown). Likewise, similar transcripts from which the poly(A) tract was missing also exhibited no interaction with the IRES (Fig. 4b).

In favour of the specificity of 3’ UTR–IRES interactions, the picornavirus SVDV 3’ UTR did not show a significant interaction with the FMDV IRES. The SVDV 3’ UTR transcript formed dimers under these conditions to a greater extent than FMDV (Fig. 4b, compare probe 3’ SVDV in lane 9 with probe 3’ UTR in lane 1). Therefore, taking the results from Figs 3 and 4 together, we concluded that the 3’ UTR–IRES interaction was dependent on a high-order structural conformation adopted by each of these complete regions.

**3’ UTR–S RNA–RNA interaction requires a poly(A) tail-induced conformation**

To map the position of residues responsible for 3’ UTR–S interactions, the 3’ UTR transcript was shortened progressively. Elimination of the poly(A) tail in construct 3’ΔΔ(A) abrogated RNA–RNA complex formation (Fig. 5). Probes SL1 and SL2 confirmed that each of these transcripts interacted with the S region, although a different retarded complex pattern was detected. The SL2 transcript, lacking the SL1 stem–loop, appeared to form one complex. However, transcript SL1, lacking the SL2 stem–loop, yielded two similarly intense, retarded complexes. As with the entire

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**Fig. 2. Interaction of the FMDV 5’ and 3’ ends is RNA concentration dependent.** (a) Unlabelled S transcript (12.5–1000 nM) was incubated with labelled 3’ UTR probe (12.5 nM) in binding buffer containing 1 mM MgCl₂ and fractionated on a 6% native gel in TBM buffer. (b) The reverse assay was performed using labelled S probe (12.5 nM) and a range concentrations of the 3’ UTR transcript (12.5–800 nM). (c) Unlabelled IRES transcript interaction (12.5–1200 nM) with labelled 3’ UTR probe. For quantitative analysis, the amount of retarded probe was normalized to the intensity of the entire lane.

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other in the absence of proteins, in a dose-dependent manner. In contrast, the efficiency of the 3’ UTR–IRES interaction was lower, with 45% of the probe retarded at saturation (Fig. 2c). In all three cases, only one retarded complex was detected.

**Specific RNA–RNA interaction between the 3’ end region and the IRES depends on the integrity of the interacting elements**

The interaction observed between the 3’ UTR sequences and the IRES region provided a mechanistic basis for the
3’ UTR sequence, elimination of the poly(A) tract in each of these transcripts impaired RNA–RNA interaction (Fig. 5). Thus, absence of the poly(A) tail in the 3’ end transcripts caused a severe decrease in the efficiency of binding. The SVDV 3’ UTR probe containing a poly(A) tail, as with the FMDV 3’ UTR, did not result in significant RNA–RNA interactions. On the basis that a poly(A) probe did not yield RNA–RNA complexes with the S region (Fig. 5), we concluded that the interaction observed between the 3’ UTR and the S region depended on the poly(A) tract but was not due to interaction with adenine residues.

**The 3’ UTR does not interact simultaneously with the IRES and S regions**

Because of the different functions attributed to the IRES and S regions (translation and replication, respectively) (Belsham & Martinez-Salas, 2004), their shared capacity to interact with the 3’ end region found here was surprising. In order to determine the possible prevalence of each one in the RNA–RNA interaction, we carried out a binding assay using combinations of S and IRES transcripts in equimolar amounts to interact with the 3’ UTR probe. No decrease in the binding capacity of either of these regions with the 3’ UTR probe in the presence of the other was observed (Fig. 6a). A higher concentration of the divalent cation did not modify this result (data not shown). Next, changes in the relative concentrations of IRES and S were used in this assay to assess their simultaneous binding efficiency to the 3’ UTR probe. As shown in Fig. 6(b), no other complexes were detected. Instead, as the concentration of one transcript was increased, the efficiency of complex formation of the transcript at the lower concentration decreased. Thus, the S and IRES regions did not interact simultaneously with the 3’ UTR under the conditions tested. This feature may have functional implications in the biology of the viral RNA, as differentially circularized RNAs may perform different roles during the virus replication cycle, one preferentially being used for translation and the other for replication.

**The S and 3’ end regions interact with a common protein**

To our knowledge, no information regarding the ability of the FMDV S region to interact with proteins is available to date. Likewise, its presumed involvement in RNA replication has not been determined precisely (Belsham & Martinez-Salas, 2004). Proteins interacting with the 3’ UTR region in a UV cross-linking assay have been reported previously (Lopez de Quinto et al., 2002). Two proteins, p120 and p47, present in HeLa and BHK-21 cell extracts, were found to interact with both the S and 3’ UTR probes (Fig. 7a). Additionally, the 3’ UTR interacted with p70 and two polypeptides of about 30 and 34 kDa. All of the cross-linked products were sensitive to proteinase K treatment. The interaction of the 3’ UTR with p70 was related directly to the presence of the poly(A) tract (Fig. 7b). In addition, p70 was recognized specifically by anti-PABP serum following immunoprecipitation of the 3’ UTR-cross-linked extracts (Fig. 7c). The anti-PABP serum has been demonstrated previously to interact with PABP (Burgui et al., 2003). No immunoprecipitation of p70 was observed with control sera raised against unrelated proteins.

To determine whether p47 and p120 interacting with both the 3’ UTR and the S region were the same factors, a competition assay was carried out. Pre-incubation of the protein extract with unlabelled 3’ UTR RNA led to a reduction in the p47 component bound to the S probe (Fig. 8a), without a significant effect on the binding capacity of p120. In the reverse assay, a similar decrease in p47 binding was observed. The mobility of this protein was coincident with a polypeptide recognized by a specific antiserum generated against PCBP1–PCBP2 (Fig. 8a), a protein previously shown to interact with the PV cloverleaf structure (Gamarnik & Andino, 1997). No competition was observed with any region of the IRES tested (Fig. 8b), coincident with a polypeptide recognized by a specific antiserum generated against PCBP1–PCBP2 (Fig. 8a), a protein previously shown to interact with the PV cloverleaf structure (Gamarnik & Andino, 1997). No competition was observed with any region of the IRES tested (Fig. 8b), without a significant effect on the binding capacity of p47. In the reverse assay, a similar decrease in p47 binding was observed. The mobility of this protein was coincident with a polypeptide recognized by a specific antiserum generated against PCBP1–PCBP2 (Fig. 8a), a protein previously shown to interact with the PV cloverleaf structure (Gamarnik & Andino, 1997).
result suggested that either the RNA–RNA interaction reorganized the structure required for binding or the binding site was occupied by RNA interactions. Either of these possibilities is compatible with the RNA–RNA interactions shown in this report.

**DISCUSSION**

The FMDV genome contains several elements in its UTRs that are well defined from a functional and structural point of view. In the 5′ UTR, the IRES element is responsible for the cap-independent translation initiation of the viral RNA (Hellen & Sarnow, 2001; Martínez-Salas et al., 2001), whereas the S region is assumed to be involved in RNA replication (Belsham & Martinez-Salas, 2004). In this study, we found two distinct long-range RNA–RNA interactions dependent on the 3′ UTR, one with the S region and another with the IRES element. To our knowledge, this is the first report of RNA–RNA interactions between the ends of a positive-strand viral genome involving two essential but functionally unrelated elements in the 5′ UTR.

Various experimental results indicated that, in the case of FMDV, IRES–3′ UTR or S–3′ UTR RNA–RNA interactions resided in different motifs. Firstly, whereas the IRES element required both 3′ UTR stem–loops, SL1 and SL2, in order to allow a physical interaction, the S region was recognized by each of the separate stem–loops. Secondly, S–3′ UTR interaction, but not IRES–3′ UTR interaction, was dependent on a structural conformation induced by the presence of the poly(A) tract. However, simultaneous binding of the S and IRES regions to the 3′ UTR was undetected in mixtures of the three transcripts, even at high RNA concentrations.

Quinto et al., 2001). We therefore concluded that p47 was a common factor interacting with both the 3′ UTR and the S region of the FMDV genome. This result led us to suggest that p47 might cooperate to bridge the 3′ end and S regions in the viral RNA.

To test this possibility, 3′ UTR and S transcripts were exposed to protein extracts after a previous RNA–RNA pre-incubation step (Fig. 8c). The simultaneous presence of these RNAs induced a small increase in the binding capacity of p47. In addition, RNA–RNA pre-incubation of S region and 3′ UTR transcripts impaired binding of p30/34. This
A peculiarity exhibited by the genome of picornaviruses belonging to different genera is that genetic hybrids carrying substitutions of functionally homologous regions show compromised infectivity to a certain extent. Thus, exchange of IRES elements between entero- and cardioviruses delays viral growth (Alexander et al., 1994; Gromeier et al., 1996), exchange of 3’ UTR sequences between PV and bovine enteroviruses or hepatitis A virus diminishes replication levels (Rohll et al., 1995) and substitution of the SVDV 3’ UTR for that of FMDV abrogates viral infectivity (Sáiz et al., 2001). This feature might be attributed to at least two different properties of the picornavirus genome. Firstly, although the genetic organization is similar (Agol, 2002), specific sequences in the respective RNAs have evolved to interact with different transacting factors, either replication- or translation-competent elements. Secondly, intramolecular interactions involving specific 3’ UTR high-order structures would not be conserved appropriately in the resulting genetic hybrid RNA. As shown in this report, the 3’ UTR of SVDV did not interact with the S or IRES regions of FMDV, consistent with the functional relevance of the RNA–RNA interactions studied. This is also in accordance with the observation that deletion of the FMDV and PV 3’ UTR severely compromises infectivity (Brown et al., 2005; Mellits et al., 1998; Sáiz et al., 2001; Todd et al., 1997). Our results therefore suggest that viral evolution might be influenced strongly by RNA–RNA intramolecular interactions, conferring phenotypic properties on the viral RNA molecule. Indeed, in spite of the high FMDV genetic variability (Carrillo et al., 2005; Domingo et al., 1992), the secondary structure of the three regulatory regions studied here was conserved.

An in silico search of nucleotide sequence complementarities among conserved residues of the 3’ UTR and the S region, potentially contributing to distant interactions, showed ten short motifs of 4–5 nt that might be responsible for this contact (data not show). It was striking that the intense IRES–3’ UTR interaction observed in vitro required the integrity of each region. This result strongly suggested that either a high-order RNA structure, as described for enteroviruses (Melchers et al., 2000; van Ooij et al., 2006), is required to offer the appropriate conformation for efficient binding, or there is a cooperative effect, since the individual stem–loops were not sufficient to stabilize the binding.

Translation and replication occur as consecutive but not simultaneous steps on the same viral RNA molecule during the picornavirus infectious cycle (Gamarnik & Andino,
UTR probes, pre-incubated for 30 min at 37 °C, were added to the UV cross-linked reaction with labelled S and 3’ UTR regions d3 and d4-5, performed as in (a). (b) Lack of competition of FMDV 1:1000 dilution of a specific anti-PCBP1/PCBP2 serum Western blot carried out with BHK-21 cell extracts using a S RNA and vice versa. The far right-hand panel shows a RNA–RNA interaction, indicating that different proteins were resolved by SDS-PAGE (10% gel).

Fig. 8. (a) 3’ UTR and S regions compete for binding to p47. A molar excess of 50-, 200- and 400-fold unlabelled 3’ UTR RNA was added to the UV cross-linked reaction with labelled S RNA and vice versa. The far right-hand panel shows a Western blot carried out with BHK-21 cell extracts using a 3’ UTR–IRES interaction. Proteins were resolved by SDS-PAGE (10% gel).

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1998; Novak & Kirkegaard, 1994). We have demonstrated that the 3’ UTR–IRES interaction occurred independently of the S–3’ UTR interaction, indicating that different residues are involved in these interactions. On the other hand, these interactions did not occur simultaneously in the same molecule. Indeed, no third complexes were detected when a wide RNA concentration range of both transcripts was added to a binding assay with a 3’ UTR probe. It is possible that a switch from translation to replication, as suggested for PV (Gamarnik & Andino, 1998), may be governed by a transition from the 3’ UTR–IRES to the 3’ UTR–S interaction during the early stages of infection. Further experiments are required to test this possibility and to determine the factors that could participate in this transition.

Genome circularization promoted essentially by a single RNA–RNA interaction has been shown recently in flaviruses (Alvarez et al., 2005). In this case, inverted terminal repeats had been noted previously (Hahn et al., 1987; You et al., 2001). Direct RNA–RNA interaction between a small number of residues in 3’ and 5’ UTR sequences has been demonstrated to control cap-independent translation initiation in RNA plant viruses (Fabian & White, 2004; Guo et al., 2001; Shen & Miller, 2004) that may be reminiscent of 5’–3’-end interaction in cap-dependent translation initiation of cellular mRNAs (Hentze, 1997).

The possibility that proteins might further stabilize RNA–RNA bridges in the FMDV genome is open. In the case of flavivirus, it has been shown that several proteins, including elongation factor 1–a, La and PTB, interact with the 3’ UTR (De Nova-Ocampo et al., 2002). A large complex of the NFAR family has been proposed to interact with the 5’ and 3’ UTRs of the pestivirus genome, possibly acting as regulators of the virus life cycle (Isken et al., 2003, 2004). In our assays, four polypeptides were shown to interact specifically with the 3’ UTR region. Currently, the nature of the p120 and p30/34 interaction with the FMDV 3’ UTR still needs to be determined. Specific p70 immunoprecipitation with anti-PABP sera, together with lack of interaction with transcripts devoid of a poly(A) tail, strongly supports the conclusion that p70 corresponds to PABP. Immunodetection assays are consistent with p47 presumably corresponding to PCBP, another candidate to interact with the poly(C) tract. Three C-rich motifs interspersed in the 3’ UTR suggest the possibility of more than one PCBP contact site. Thus, C-rich motifs may be present in the S region (data not shown). There is no apparent conservation between the PV cloverleaf structure and the S region of FMDV. However, some of the C-rich motifs resemble the ACCCCA primary sequence shown to be the preferential binding site of PCBP (Gamarnik & Andino, 1997; Parsley et al., 1997). No competition was observed with the central domain of the FMDV IRES, previously inferred to interact with PCBP from RNA depletion assays (Stassinopoulos & Belshaw, 2001). Thus, binding of PCBP to the S and 3’ UTR FMDV regions displayed a higher affinity than that to the IRES element. It therefore remains possible that this protein interacts with the most distal regions of the FMDV genome, probably reinforcing the RNA–RNA S–3’ UTR interaction demonstrated here.

The dual involvement of the 3’ UTR in controlling viral RNA translation and virus replication highlights the relevance of this region in the infectious virus life
cycle, making it a suitable candidate for targeted antiviral therapy.

ACKNOWLEDGEMENTS

We are grateful to R. Andino and A. Nieto for the anti-PCBP and anti-PABP antisera, respectively; Silvia Gómez for early contributions to the SL1 and SL2 constructs and O. Fernández-Miragall for advice with the RNA–RNA interaction assays. M. S. is the holder of a grant from ‘Programa Ramón y Cajal’ from the Spanish Ministry of Education and Science. This work was supported by grants BMC2002-00983, BFU2005-00948 to E. M.-S., RTA03-201 to M. S. and by an Institutional grant from Fundación Ramón Areces.

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